The effect of combined application of TGFβ-1, BMP-2, and COLLOSS® E on the development of bone marrow derived osteoblast-like cells *in vitro*

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Abstract: This study investigated the combined application of Transforming Growth Factor β-1 (TGFβ-1) and Bone Morphogenetic Protein-2 (BMP-2) to stimulate osteogenic expression *in vitro*. TGFβ-1 and BMP-2 fulfill specific roles in the formation of new bone. COLLOSS® E, a bone-derived collagen product containing a variety of naturally occurring growth factors, was also used. Growth factors were administered to osteoblast-like cells from rat bone marrow (RBM). Proliferation and differentiation were monitored up to 24 days, by measuring total DNA content, alkaline phosphatase activity, and calcium content. Genetic expression of a set of differentiation markers at day 7 was measured by Q-PCR. Adding BMP-2 alone induced high proliferation rates, compared to the growth factor supplemented groups, and it induced high differentiation rates, compared to the control group. Adding TGFβ-1 combined with BMP-2, TGFβ-1 alone, or COLLOSS® E resulted in a significant decrease in proliferation rate, but an increase in differentiation rate, compared to the control group. Additive or synergistic effects of application of TGFβ-1 and BMP-2 were not observed. The observed effects of COLLOSS® E mainly resembled those of TGFβ-1 application alone. It can be concluded that BMP-2 is the most suitable candidate for osteogenic stimulation of RBM cells in these settings. © 2007 Wiley Periodicals, Inc. J Biomed Mater Res 86A: 788–795, 2008

Key words: cell culture; osteoblast differentiation; bone morphogenetic protein-2; transforming growth factor β-1

INTRODUCTION

For the regeneration of large bone defects resulting from trauma, pathology, or congenital malformations, synthetic bone substituting materials have been developed, some of which incorporate one or more of the biological features of an autologous bone graft. These features include osteoconductive and osteoinductive properties, in addition to viable osteoprogenitor cells.[1] Bone tissue engineering techniques can be used to grow osteoprogenitor cells on synthetic or natural scaffolds,[2,3] while growth factors like the Insulin-like Growth Factors (IGFs) and members of the Transforming Growth Factor-β (TGF-β) superfamily, TGFβ-1, TGFβ-2, TGFβ-3, BMP-1, BMP-2, and BMP-7 (aka OP-1), are able to grant scaffolds osteoinductive properties.[1]

One of the TGF-β superfamily members is TGFβ-1, which is expressed at high levels during bone development, growth, and healing. Osteoblasts produce TGFβ-1, which is incorporated into mineralized bone matrix.[1] *In vitro*, TGFβ-1 has been observed to both inhibit and stimulate osteoblastic cell proliferation depending on TGFβ-1 concentration, cell density, and the stage of differentiation.[1,4,5] *In vivo*, TGFβ-1 appears to play a decisive role in the early phase of bone synthesis.[6]

Bone morphogenetic proteins (BMPs) form a unique group of proteins within the TGF-β superfamily. BMP-2 promotes differentiation of osteoprogenitor cells into osteoblasts and matrix formation, thus making it a suitable growth factor to use for bone tissue engineering.[4,7–10] The expression of BMP-2 and TGFβ-1 is thought to be regulated by a positive feedback mechanism of these two growth factors.[1]

Although recombinant growth factors are potent, the high expense poses a problem for clinical treat-

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ment. To solve this problem, alternative sources of growth factors are also examined, for instance, COLLOSS® E. This is a lyophilized complex of extracellular-matrix proteins extracted from (equine) long bones. The preparatory methods are similar to those originally used by Urist and Strates to isolate BMPs.[11] The main constituents of COLLOSS® E are collagen type I chains in combination with TGFβ-1, BMP-2, BMP-7, VEGF, IGF-1, TGFβ-2, and BMP-3 and several other, not yet determined, growth factors. COLLOSS® E is proven to be osteoinductive in an *in vivo* situation.[12–16] However, the use of COLLOSS® E as a stimulus for mesenchymal stem cells for a tissue engineering approach is not yet described.

The aim of this study was to compare the effects of different growth factors at various concentrations, and in different combinations, on rat bone marrow (RBM) cells *in vitro.* TGFβ-1, BMP-7, and BMP-2 are commercially available; however, in this study only TGFβ-1 and BMP-2 are used to reduce parameters. It is hypothesized that TGFβ-1 mainly stimulates the proliferation, and BMP-2 the differentiation of the cells, whereas together they obtain an additive or even synergistic effect on cell growth and differentiation. COLLOSS® E is expected to function likewise as TGFβ-1 combined with BMP-2.

Therefore, in the current study setup RBM cells were seeded onto 24-well dishes and cultured in medium for 24 days, with added growth factors for the first 8 days. The effect of the growth factors was evaluated by DNA content assay, ALP activity assay, and calcium assay and by scanning electron microscopy (SEM) on days 1, 4, 7, 16, and 24. In addition, on day 7, the effect of the growth factors on mRNA expression of osteocalcin, collagen type I, alkaline phosphatase, and bone sialoprotein was evaluated with quantitative PCR.

MATERIALS AND METHODS

Osteoblast-like cell isolation and culture

RBM cells were isolated and cultured as described by Maniatopoulos et al.[2] Briefly, RBM cells were obtained from the femora of two 40- to 43-day-old male Wistar WU rats. The femora were washed three times in alpha Minimal Essential Medium (α-MEM; Gibco BRL, Life Technologies B.V. Breda, The Netherlands) supplemented with 0.5 mg/mL gentamycin (Gibco) and 3 μg/mL fungizone (Gibco). Epiphyses were cut off and diaphyses flushed out with 15 mL culture medium [α-MEM supplemented with 10% fetal calf serum (Gibco), 50 μg/mL gentamycin, 50 μg/mL ascorbic acid (Sigma Chemical Co., St. Louis, MO)], 10 m*M* Na-β-glycerophosphate (Sigma), and 10^{-8}*M* Dexamethasone (Sigma).

To obtain osteoblast-like cells, the RBM suspension was divided over six culture flasks (75 cm^2), and incubated in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. The following day, culture medium was refreshed to remove all nonadherent cells. After 7 days of primary culture, cells were detached using trypsin/EDTA [0.25% (w/v) trypsin/0.02% EDTA], resuspended, and seeded at 10,000 cells/cm^2 onto 24-well plates (Greiner, Bio-One, Alphen aan den Rijn, The Netherlands). After 4 h, the culture medium was replaced with fresh culture medium with added growth factors (Table I). The concentrations of TGFβ-1 and BMP-2 were determined from previous literature.[8,9,17] COLLOSS® E is known to contain 38.62 ng/mg TGFβ-1 and 4.12 ng/mg BMP-2, as well as a multiplicity of other growth factors. All samples for each condition and each time point were present threefold and the entire experiment was performed twice.

TABLE I
Concentrations and Combinations of Growth Factors Used in This Experiment in ng/mL (TGFβ-1 and BMP-2) and in μg/mL (COLLOSS® E)

Group	TGF β-1 (ng/mL)	BMP-2 (ng/mL)	COLLOSS® E (μg/mL)
1	5.0 (first 8 days)		
2		100.0 (first 8 days)	
3	5.0 (first 4 days)	100.0 (days 4–8)	
4	5.0 (first 8 days)	100.0 (first 8 days)	
5			2.5 (first 8 days)
6			5.0(first 8 days)
7			10.0 (first 8 days)
8 (control)			

DNA content

After 1, 4, 7, 16, and 24 days of culture, total DNA content was determined to obtain information about cellular proliferation, since the DNA content directly correlates to the amount of the cells. Medium was removed and the cell layer washed twice with PBS. Subsequent addition of 1 mL MilliQ deionised water to each well and sonication for 10 min lysed the cells. Samples were stored at −20°C until further use.

For analysis, a PicoGreen dsDNS Quantation Kit (Molecular Probes, Leiden, The Netherlands) was used. A standard curve was generated using serial dilutions of lambda DNA ranging from 0 to 2000 ng/mL. Briefly, 100 μL of sample and 100 μL PicoGreen working solution were added to a 96-well plate (Greiner). The plate was incubated at room temperature in the dark for 5 min. The plate was then read at 480–520 nm and the DNA content was determined with the aid of the standard curve. Samples and standards were assayed in duplicate.

Alkaline phosphatase activity

The ALP activity was measured after 1, 4, 7, 16, and 24 days, with the same samples as used in the DNA-content

TABLE II
Amplification Primers Used for the Q-PCR of Osteoblast-Like Cells

	Amplification Primer 1 (Forward)	Amplification Primer 2 (Reverse)
Osteocalcin (OC)	GGCTTCCAGGACGCCTACA	CATGCCCTAAACGGTGGTG
Collagen type I (Col I)	TGGAATCTTGGATGGTTTGGA	GCTGTAAACGTGGAAGCAAGG
Alkaline phosphatase (ALP)	GCTTCACGGCATCCATGAG	GAGGCATACGCCATGACGT
Bone sialoprotein (BSP)	ACTTCCCTTCGCAAGCTTAGG	AAACTTCCCGCGTATGTTGG
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	GCCTAAATGATACCCCACCGT	GCTGGCACTGCACAAGAAGA

assay. For the assay, 80 μL of sample and 20 μL of buffer solution [5 m*M* $MgCl_2$, 0.5*M* 2-amino-2methyl-1-propanol (Sigma)] were added to 96-well plates. Subsequently, 100 μL of substrate solution [5 m*M* paranitrophenylphosphate (Sigma)] was added and the plate was incubated for 1 h at 37°C. The reaction was stopped by the addition of 100 μL of 0.3*M* NaOH and finally, the plate was read at 405 nm. With the aid of a standard curve [4-nitrophenol (4-NP; Sigma)], serial dilutions ranging from 0 to 25 n*M*, the ALP activity was determined in nmol 4-NP produced per hour per nanogram DNA. Samples and standards were assayed in duplicate.

Calcium

To obtain information about mineralized matrix formation, the calcium (Ca) content was determined after 7, 16, and 24 days of culturing. The samples originated from the same 24-well plates as used for the DNA content and ALP activity assays. Ca content was determined using the ortho-cresolphtalein complexone [OCPC (Sigma)] method. The 24-well plates were rinsed twice with PBS followed by the addition of 500 μL acetic acid (0.5*N*) and overnight incubation at room temperature. Samples were frozen at −20°C until further use.

OCPC solution was prepared by the addition of 80 mg of OCPC to 75 mL of demineralized H_2O with 0.5 mL KOH (1*M*) and 0.5 mL acetic acid (0.5*N*). To prepare the sample solution, 5 mL of OCPC solution was added to 5 mL 14.8*M* ethanolamine–boric acid buffer (pH 11), 2 mL of 8-hydroxyquinoline (5 g in 100 mL 95% ethanol), and 88 mL of demineralized water. Three-hundred microliters of this sample solution was added to 10 μL of sample. The plate was incubated at room temperature for 10 min and read at 575 nm using a microplate reader. A standard curve was made from serial dilutions of $CaCl_2$, ranging from 1–200 μg/mL. Samples and standards were assayed in duplicate.

Realtime-PCR

First, RNA was isolated from the cells after 7 days of culture (six wells were pooled), with the RNeasy isolation kit (Qiagen Benelux B.V., Venlo, The Netherlands). Subsequently, a reverse transcriptase (RT) reaction was performed by supplementing 1 μg of total RNA, 1 μL (50–250 ng) of random primers, and 1 μL dNTP mix (10 m*M* each) in a total volume of 15 μL RNase-free water. The mixture was heated to 65°C for 5 min and quickly chilled on ice. The contents of the tube were collected by brief centrifugation and 4 μL 5× First Strand Buffer (250 m*M* Tris-Cl, pH 8.3, 375 m*M* KCl, 15 m*M* $MgCl_2$) and 2 μL 0.1*M* DDT were added. This mixture was incubated for 10 min at 25°C, followed by the addition of 1 μL (200 units) of SUPERSCRIPT II and incubation for 50 min at 42°C. The reaction was inactivated by heating the mixture at 70°C for 15 min. The cDNA was used as a template in the real-time polymerase chain reaction (Q-PCR).

iQ SYBR Green Supermix [12.5 μL; 2× mix contains 100 m*M* KCl, 40 m*M* Tris-HCl, pH 8.4, 0.4 m*M* each dNTP (dATP, dCTP, dGTP, dTTP), 50 U/mL iTaq DNA polymerase, 6 m*M* $MgCl_2$, SYBR Green I, 20 n*M* fluorescein, and stabilizers; BioRad, Hemel Hempstead, UK], 1.5 μL forward amplification primer (10 μ*M*; see Table II), 1.5 μL reverse amplification primer (10 μ*M*; see Table II), 2 μL cDNA, and 7.5 μL RNase-free water were added to a PCR reaction plate to a final volume of 25 μL per sample. The contents of the plate were mixed gently and collected by brief centrifugation.

The plate was placed in the Q-PCR MyiQ Single-color Real Time detection system and the reaction started after a heat reaction of 94°C for 3 min. The measurements were performed by the following calculations, with GAPDH serving as a control:

1. $\Delta C_T = C_{T,\text{gene of interest}} - C_{T,GAPDH}$
2. $\Delta\Delta C_T = \Delta C_{T,\text{sample}} - \Delta C_{T,\text{control}}$
3. $2^{-\Delta\Delta CT}$

Scanning electron microscopy

Samples were taken after 1, 4, 7, 16, and 24 days, and washed three times with PBS. Fixation of the cells was carried out for 5 min in 2% glutaraldehyde. Then, the substrates were washed for 5 min with 0.1*M* sodium–cacodylate buffer (pH 7.4), dehydrated in a graded series of ethanol, and dried with tetramethylsilane. The substrates were sputter-coated with gold and examined using a Jeol 6310 SEM.

Statistical analysis

The complete experiment was performed twice. Every sample was present threefold and measured in duplicate. Statistical analysis was performed using an ANOVA with

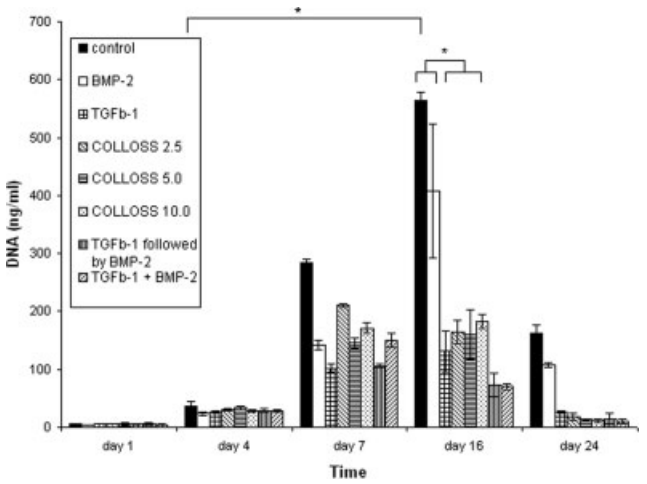

Figure 1. Cell proliferation curve by a total DNA measurement; results are in ng/mL.

post-hoc Tukey test. Calculations were performed in InStat software (v 3.05 Graphpad).

RESULTS

In general, the results of all assays were largely comparable between both the individual experiments. All depicted results are derived from the second experiment.

DNA content

The results from the DNA assay are shown in Figure 1. The control group gave a significant increase in DNA content until day 16. After day 16, a decrease in DNA content was observed. A similar growth pattern like that of the control group was seen for all other groups. However, the combined TGFβ-1/BMP-2, and the 2.5 μg/mL COLLOSS® E groups reached maximum values at earlier time points. Following the control group, the highest DNA content was observed for the BMP-2 group. The TGFβ-1 combined with BMP-2 groups gave the lowest contents. The DNA content of the COLLOSS® E and the TGFβ-1 groups was just above the TGFβ-1 combined with BMP-2 groups and peaked around day 16. At day 16 a significant difference was observed between the control and BMP-2 group and the other groups.

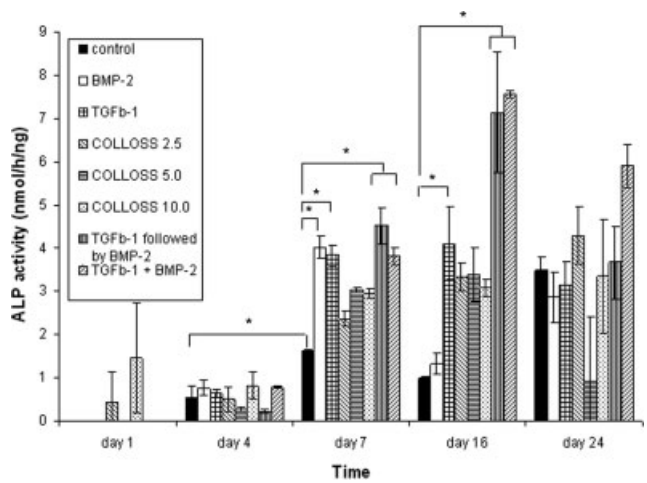

Figure 2. ALP activity measurement results. The ALP activity was determined in nmol 4-NP produced per hour per nanogram DNA (nmol/h/μg). ALP is an early differentiation marker and correlates to the differentiation of the cells.

Alkaline phosphatase activity

Results of the ALP activity measurement are depicted in Figure 2. The control group showed a significant increase in activity with time, with the highest measurements between days 7 and 16. On days 7 and 16, a significant upregulation of ALP activity, with a maximum around day 16, was observed for the TGFβ-1 combined with BMP-2 groups when compared to the control group. The ALP activities of the TGFβ-1 and COLLOSS® E groups lay below those of the TGFβ-1 combined with BMP-2 groups; the upregulation of TGFβ-1 was significant on days 7 and 16 compared to the control group. They reached their maxima around day 16. A maximum peak in ALP activity was seen early for the BMP-2 group at day 7 and at that time point, the ALP activity was significantly upregulated when compared with the control group.

Calcium

Results from the Ca measurement are depicted in Figure 3. No calcium content was detectable before

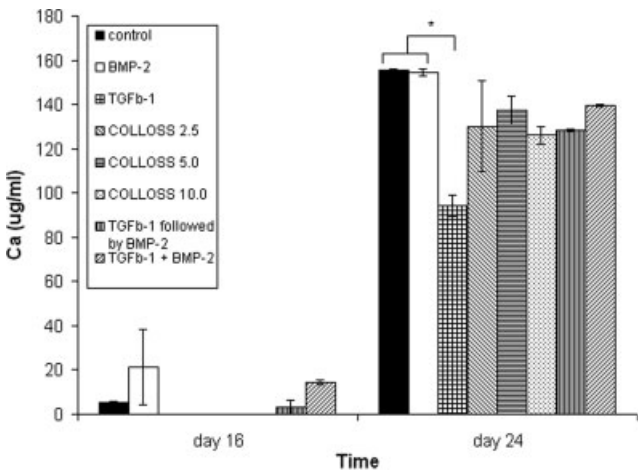

Figure 3. Concentration of calcium in μg/mL. Calcium is a late differentiation marker and correlates to the late differentiation of the cells.

TABLE III
Q-PCR Results (in Percentages)

	OC (%)	Col I (%)	ALP (%)	BSP (%)
TGFβ-1	49	181	51.80	46
BMP-2	167.00	NA	125.70	343.40
TGFβ-1 followed by BMP-2	78.50	NA	254.90	783.50
TGFβ-1 + BMP-2	76.80	111	459.50	135.70
COLLOSS® E, 2.5 μg/ml	22.50	NA	99.30	85.30
COLLOSS® E, 5.0 μg/ml	17.10	NA	44.40	32.50
COLLOSS® E, 10.0 μg/ml	8.20	85	77.40	41.80

Samples are compared to the control sample and corrected for the household gene—Glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

day 16. On day 16, the content started to increase significantly upto day 24 for the control group. The measurements of the BMP-2 group seemed to increase at an earlier time point. The TGFβ-1 group showed no mineralization at day 16 and a significantly lower value at day 24, compared to the control group. The calcium content of the TGFβ-1 combined with BMP-2 and the COLLOSS® E groups were between the control and TGFβ-1 group, with little mineralization for the TGFβ-1 combined with BMP-2 groups and no mineralization for the COLLOSS® E groups at day 16. On day 24 the calcium content of the control and BMP-2 group are similar, and seems to be lower in the other groups. However, this difference is not significant except for the TGFβ-1 group.

Realtime-PCR

The quantative PCR results are depicted in Table III. The TGFβ-1 group showed an increase in mRNA for Col I, whereas all other differentiation markers (ALP, OC, and BSP) were down-regulated. The BMP-2 group gave exactly opposite results; all differentiation markers were upregulated, however, Col I could not be detected. The TGFβ-1 + BMP-2 group showed a small decrease in OC and an increase in BSP and especially in ALP. The TGFβ-1, followed by BMP-2 group, also showed a small decrease in OC and high increases in ALP and especially BSP. The COLLOSS® E groups in general showed a down-regulation of all differentiation markers, and mostly resembled the TGFβ-1 group.

Scanning electron microscopy

Representative scanning electron micrographs are depicted in Figure 4(A,B) (control samples, days 4 and 24), Figure 5(A,B) (BMP-2 samples, days 4 and 24), and Figure 6(A,B) (COLLOSS® E, 10.0 μg/mL samples, days 4 and 24). The control group showed a pattern of cell growth and differentiation starting with cell attachment, spreading, and proliferation at days 1–4, matrix formation around days 7–16, and calcification of the matrix after day 16, which was expected. This pattern of growth and differentiation was also observed for all the other groups. However, the TGFβ-1 combined with BMP-2 groups started to calcify the matrix before day 16 and as a general remark, it could be said that the BMP-2 group displayed a confluent monolayer and an even distribution of matrix like the control group, whereas all other groups showed patches of cells with matrix formation and calcification.

DISCUSSION

Expansion of knowledge concerning growth factors is a necessity when preparing osteoinductive

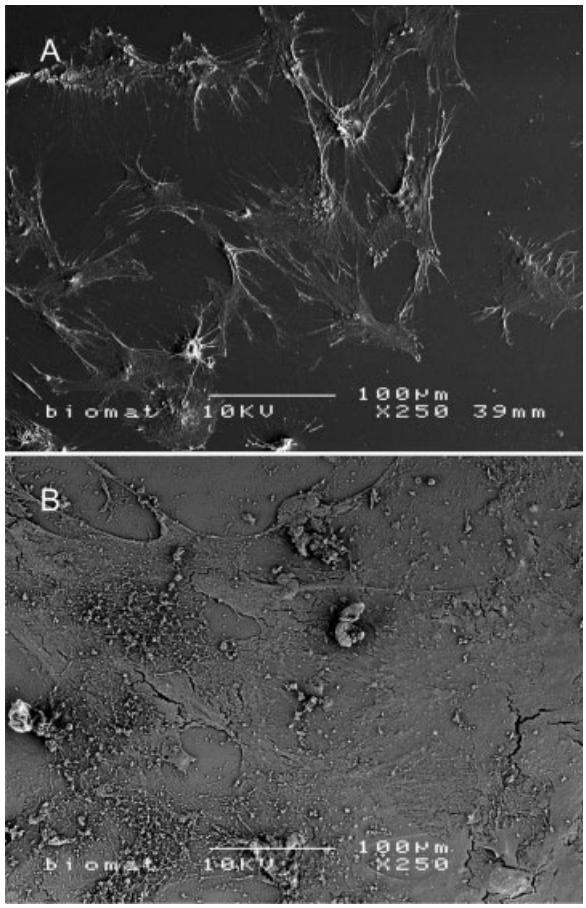

Figure 4. A, B: Scanning electron micrographs of the control samples at days 4 (A) and 24 (B) at ×250 magnification. The control samples show cell attachment and spreading on day 4 and calcification of the extracellular matrix at day 24.

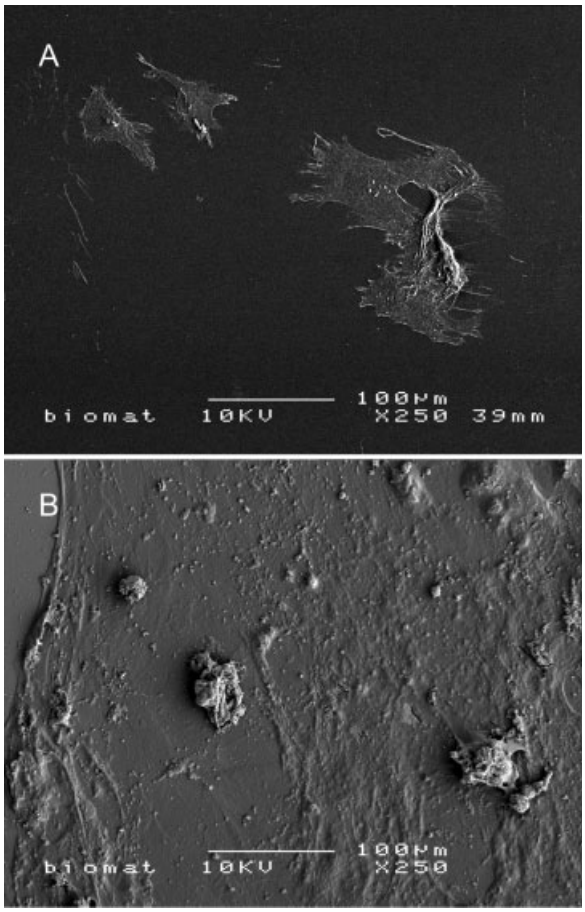

Figure 5. A, B: Scanning electron micrographs of the BMP-2 samples at days 4 (A) and 24 (B) at ×250 magnification. Note, the extensive cell attachment and spreading on day 4 as well as the calcification of the extracellular matrix at day 24, as also seen for the control samples [Fig. 4(A,B)].

scaffolds. In the current investigation, a number of relevant growth factors were examined individually and in combinations. The focus of this project was on the effects of the growth factors on proliferation and differentiation of osteoblast-like cells. Good proliferation and differentiation rates were seen after BMP-2 application and increased differentiation rates were observed for the TGFβ-1 combined with BMP-2 groups.

Regarding our study setup, some variance in proliferation and differentiation onset of the cells, between the first and the second experiment was observed. However, although the differentiation of the cells, matrix formation, and calcification started somewhat later in the first experiment, the growth and differentiation curve between all groups corresponded in both experiments. Since data were reproducible, valid conclusions can be made. The phenomenon of differing growth and differentiation rates between experiments is due to the use of primary cells; earlier studies already reported about the use of these primary cell lines, which did not produce the exact same results.[9,18] Another technical remark has to be made on the addition of the growth factors. All growth factors except BMP-2, had a marked effect on the morphology of the cell layer, in comparison to the control group. The cell layer was always less confluent and cells appeared more rounded. This effect was most markedly visible in the COLLOSS® E groups. COLLOSS® E contains high concentrations of TGFβ-1, compared to the other growth factors present in COLLOSS® E, which is known to influence the morphology and attachment of bone-like cells. Possibly, this effect is even enhanced by other, yet undetermined growth factors present in COLLOSS® E.

The decrease in DNA content, that is seen in all groups after day 16, happens when these cells are cultured for a period longer than 16 days.[19] The reason for this lies in the fact that the cells have differ-

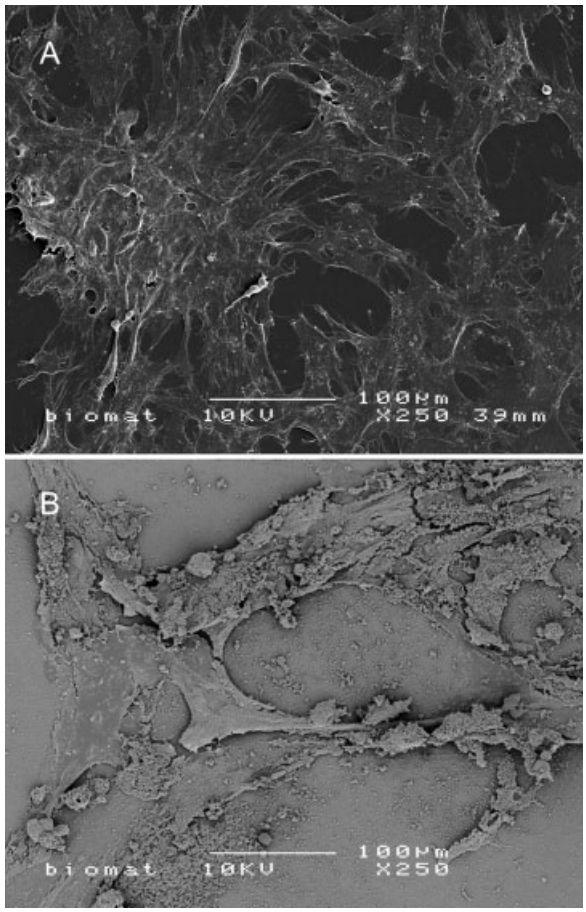

Figure 6. A, B: Scanning electron micrographs of the COLLOSS® E 10.0 µg/mL samples at days 4 (A) and 24 (B) at ×250 magnification. This group showed a cell attachment and spreading on day 4 and calcification of the extracellular matrix at day 24, as also seen for the control samples [Fig. 4(A,B)].

entiated by this time point and therefore do not proliferate anymore, combined with a lower efficiency of DNA extraction, because of embedding of the cells in the extracellular matrix.

Earlier studies suggest that BMP-2 especially stimulates the differentiation of osteoblast-like cells.[7–9] This theory is supported by the early significant upregulation of ALP activity and the upregulation of differentiation markers in the quantitative-PCR at day 7 and the high calcium content that was found at day 24. Also, the initial cell seeding density can significantly influence the final behavior of the cell cultures. For instance, in the study of van den Dolder et al., 20,000 cells/cm^2 were used, which is twice as high as the concentration used in this study. The effects of growth factors are always related to the number and differentiation stage of the cells.

The proliferation results of the cells stimulated with BMP-2 were significantly higher than those of the other groups that received growth factors, however they were always lower than the control group. A possible explanation for this phenomenon is the stimulation of differentiation of the cells by BMP-2, which causes an earlier start of differentiation of the cells that would otherwise proliferate for a longer time period.

In contrast to BMP-2, literature is more divided about the effect of TGFβ-1 on osteoblast-like cells. Some investigations show that TGFβ-1 stimulates cell proliferation and inhibits differentiation,[20,21] whereas in other articles exactly the opposite is reported.[17,22,23] These contradictory findings can also be due to differences in cell concentration and origin.[9] In our study, differentiation was stimulated by the addition of TGFβ-1, whereas proliferation was decreased.

Many growth factors play a role during the bone healing process. However, the interaction between these growth factors is not completely understood and not much information is available about the combined effect of growth factors *in vitro*. This was the reason that in our study also, combinations of growth factors were examined. The addition of TGFβ-1 and BMP-2 at the same time or followed by each other was supposed to induce synergistic effects on matrix formation and calcification. Although the ALP-activity measurements as well as the quantative PCR measurements of ALP and BSP indicated an increased cell differentiation, the complimentary use of two factors did finally not result in a confluent layer with a high degree of calcification. This can be due to the low proliferation of the cells during the initial incubation phase.

Interestingly, almost no differences were found between the COLLOSS® E groups. Apparently, all used concentrations are in the same order of magnitude and therefore exhibit similar effects.

The effect of COLLOSS® E was hypothesized to correspond to the effect of TGFβ-1 combined with BMP-2, since COLLOSS® E contains both growth factors, although, some differences can be expected, since the growth factors in COLLOSS® E are bound to collagen. The concentrations of TGFβ-1 and BMP-2 present in COLLOSS® E are also lower than those of the TGFβ-1 and BMP-2 groups in this study, however, multiple other factors present in COLLOSS® E could possibly compensate for these differences in concentration.[24] In contrast to our initial suggestion, cell proliferation was higher, and differentiation lower than seen in the TGFβ-1 combined with BMP-2 group. Evidently, this is due to a large difference in concentration balance between growth factors in the COLLOSS® E material, as recently it has been shown that 38.62 ± 13.57 ng/mg TGFβ-1 and 4.12 ± 1.32 ng/mg BMP-2 is present in COLLOSS® E. Apparently, the high concentration of TGFβ-1 overrules the effect of BMP-2. The differences, as found between the TGFβ-1 combined with BMP-2 and the COLLOSS® E groups, can even be enhanced because numerous other growth factors like VEGF, IGF-1, TGFβ-2, BMP-7, and BMP-3 are present in COLLOSS® E. Direct or indirect effects of these growth factors on the behavior of osteoblasts are proven in earlier studies. The direct effect of VEGF, IGF-1, or TGFβ-2 on the proliferation of osteoprogenitor cells is unknown, but a direct positive effect on the differentiation of osteogenic cells was confirmed for VEGF, TGFβ-2,[25,26] and IGF-1.[1,27] BMP-7 promotes proliferation as well as differentiation of osteoblastic cells and stimulates bone apposition in certain *in vivo* situations.[28,29] Although all these growth factors have a stimulatory effect on bone formation, BMP-3 has an inhibitory effect on other BMPs. BMP-3 is a receptor antagonist that competes for common signaling pathway components in the BMP signaling pathway, thereby inhibiting bone formation.[30,31] Therefore, differentiation of the RBM cells can be very well inhibited by BMP-3.

In summary, the aim of the current study was to examine the effects of different potential cell and bone-like matrix stimulating growth factors on osteoblast-like cells. Increased proliferation, compared to the groups that received growth factors, and differentiation rates were seen for cell cultures supplemented with BMP-2. The addition of TGFβ-1 combined with BMP-2, TGFβ-1, and all COLLOSS® E to the cell culture medium resulted in a decrease in proliferation rate and an increase in differentiation rate. On basis of these findings, it can be concluded that, in agreement with our initial hypothesis, BMP-2 is the most suitable candidate of these (combinations of) growth factors for the early osteogenic stimulation of RBM cells in the used experimental settings. In disagreement with our initial hypothesis,

additive or synergistic effects of mutual or successive application of TGFβ-1 and BMP-2 were not observed in this cell culture model and COLLOSS® E did not show effects resembling those of combined TGFβ-1 and BMP-2. COLLOSS® E showed effects that mostly resembled the addition of TGFβ-1, probably caused by the fact that TGFβ-1 is the predominant growth factor component present in COLLOSS® E, although the potential effects of other growth factors present in COLLOSS® E cannot be excluded.

OSSACUR AG provided COLLOSS® E. Scanning electron microscopy was performed at the Microscopic Imaging Centre (MIC) of the Nijmegen Centre for Molecular Life Sciences (NCMLS), the Netherlands.

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